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# The stringent response in *Streptomyces coelicolor* A3(2)

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## Summary

**The stringent response was elicited in the antibiotic producer *Streptomyces coelicolor* A3(2) either by amino acid depletion (nutritional shiftdown) or by the addition of serine hydroxamate; both led to increased levels of ppGpp and to a reduction in transcription from the four promoters of the *rrnD* rRNA gene set. Analysis of untreated batch cultures revealed elevated ppGpp levels at the end of exponential growth, preceding the onset of antibiotic production. The effect of provoking the stringent response on antibiotic production in exponentially growing cultures was assessed by S1 nuclease mapping of *actIII*, an early gene of the actinorhodin biosynthetic cluster. Expression of *actIII* occurred after nutritional shiftdown, but not after treatment with serine hydroxamate. Although the need for ppGpp in triggering antibiotic production remains equivocal, ppGpp synthesis alone does not appear to be sufficient to initiate secondary metabolism in *S. coelicolor* A3(2).**

## Introduction

In *Escherichia coli*, depletion of amino acids leads to the stringent response (for a review see Cashel and Rudd, 1987), which is characterized by an immediate reduction in the overall rate of RNA synthesis apparently mediated by ppGpp (guanosine 5′-diphosphate-3′-diphosphate). This nucleotide is synthesized by a ribosome-associated enzyme, [p]ppGpp synthetase I (also known as 'stringent factor') encoded by the *relA* gene, either via pppGpp from GTP and ATP or directly from GDP and ATP, and is made when uncharged codon-specific tRNA molecules bind to the ribosomal A site. During the stringent response, complex changes in the pattern of gene expression occur; transcription of stable RNA genes is immediately reduced, and there is a simultaneous increase in the expression of, for example, amino acid biosynthetic genes, of which the *his* operon is a well-studied example (Stephens *et al.*, 1975; Shand *et al.*, 1989). There is also considerable evidence to show that ppGpp plays a central role in growth rate control (Sarubbi *et al.*, 1988). Several mutations can reduce ppGpp formation and result in continued RNA synthesis during amino acid starvation, the so-called 'relaxed' phenotype. In *relA* mutants [p]ppGpp synthetase I is affected, resulting in little or no ppGpp formation after amino acid starvation. In *relC* mutants, an alteration in, or the complete lack of, ribosomal protein L11 prevents ppGpp formation by the synthetase, although at least in some cases the enzyme can still bind to the ribosome. ppGpp formation, and the apparently associated growth rate control, can occur also via a *relA*-independent pathway (Friesen *et al.*, 1978; Atherley, 1979) that responds to carbon and energy source depletion (Cashel and Rudd, 1987).

The stringent response and ppGpp formation occur in various *Streptomyces* species (Riesenberg *et al.*, 1984; Ochi, 1986; 1987a,b,c). The latter author isolated relaxed mutants, apparently analogous to *relC* mutants (Cashel and Rudd, 1987), that were deficient in antibiotic production. This suggested a possible role for the stringent response and ppGpp in determining the onset of secondary metabolism in *Streptomyces* and indirectly in promoting morphological differentiation (Ochi, 1986; 1988). The observation that antibiotic production is usually limited to cultures in stationary phase or growing at low growth rates (Demain, 1989), would at least be consistent with the apparent role played by ppGpp in growth-rate control in *Escherichia coli*.

In order to investigate these aspects of the stringent response in more detail, we have begun a study in the genetically characterized strain *Streptomyces coelicolor* A3(2). In this work we describe the effects of inducing the stringent response on intracellular nucleotide concentrations and on the transcription of a rRNA gene set (*rrnD*; Baylis and Bibb, 1988a) and of a gene involved in the biosynthesis of the antibiotic actinorhodin (*actIII*; Hallam *et al.*, 1988). The *rrnD* gene set shows a complex transcriptional arrangement and is expressed from four promoters with potentially interesting sequence heterogeneity (Baylis and Bibb, 1988b), conceivably reflecting different mechanisms of transcriptional control.

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## Results

### *Induction of the stringent response in* S. coelicolor *A3(2) by serine hydroxamate*

Serine hydroxamate (SHX), a structural analogue of L-serine, provokes the stringent response in *E. coli* by acting as a competitive inhibitor of seryl-tRNA synthetase (Tosa and Pizer, 1971), thus leading to the accumulation of uncharged $tRNA^{ser}$. SHX triggers the stringent response in *Streptomyces hygroscopicus* (Riesenberg *et al.*, 1984), presumably by the same mechanism. Two different concentrations of SHX were added to exponentially growing cultures ($OD_{450\,nm}$ = 0.35) of *S. coelicolor* A3(2) strain M145 and the effects on growth rate, nucleotide-pool levels and rRNA transcription were determined. After addition of the analogue, the cultures continued to grow exponentially but with a reduced growth rate. The higher SHX concentration (50 mM) increased the doubling time from 2.3 to 17 h, and the lower concentration (25 mM) gave a doubling time of 5.1 h. In contrast to cultures subjected to nutritional shiftdown (see below), growth continued for approximately two generations before the cultures entered stationary phase and reached the same optical density as control cultures.

Addition of SHX provoked the transient accumulation of ppGpp, giving maximal concentrations, within 15 min, of 47 and 75 pmol $mg^{-1}$ dry weight with final concentrations of 25 mM and 50 mM SHX, respectively (Fig. 1A). Sixty minutes after addition of SHX, the ppGpp concentrations had fallen to 6 and 11 pmol $mg^{-1}$ dry weight, respectively, significantly above the level found in control cultures, in which ppGpp was not detectable (the limit of detection was 3 pmol $mg^{-1}$ dry weight). The intracellular concentrations of ATP, UTP, CTP and GTP fell rapidly after the addition of 25 mM SHX (Fig. 1B); the decreases in GTP and UTP levels were particularly pronounced. Similar changes were observed for the higher SHX concentration, where the nucleoside triphosphate concentrations dropped to even lower levels. Protein synthesis, estimated by the incorporation of [$^{14}$C]-leucine into trichloroacetic acid (TCA)-precipitable material, was also severely reduced in both cases.

The accumulation of ppGpp was accompanied by an immediate reduction in the overall rate of RNA synthesis (Fig. 2). Although the amount of data is limited, there is a positive correlation between the level of ppGpp accumulation, the reduction in the overall rate of RNA synthesis and the decrease in growth rate.

The decline in overall RNA synthesis during the stringent response in *E. coli* is mainly accounted for by a drastic reduction in the synthesis of stable RNA (Gallant, 1979). All seven rRNA operons of *E. coli* are expressed from two promoters, and in at least one of these operons, *rrnA*, only the major promoter, p1, is under negative stringent control, while the weaker p2 promoter is constitutively expressed (Sarmientos *et al.*, 1983). The *rrnD* gene set of *S. coelicolor* A3(2) is transcribed from four different promoters (p1–p4) which can be grouped into two classes. p1 and p2 possess −10 and −35 regions that resemble the consensus sequences for the major class of prokaryotic promoters, while p3 and p4, which show extensive similarity to each other between −41 and +3, lack typical −35 regions (Baylis and Bibb, 1988b). The consequences of inducing the stringent response on transcription from each of the four *rrnD* promoters was studied by S1 nuclease mapping in conditions of probe excess to discern any possible differential effects on promoter utilization. (Earlier S1 nuclease mapping experiments with the *rrnD* gene set were conducted under conditions of probe limitation (Baylis and Bibb, 1988b), and did not reflect the true relative levels of the different

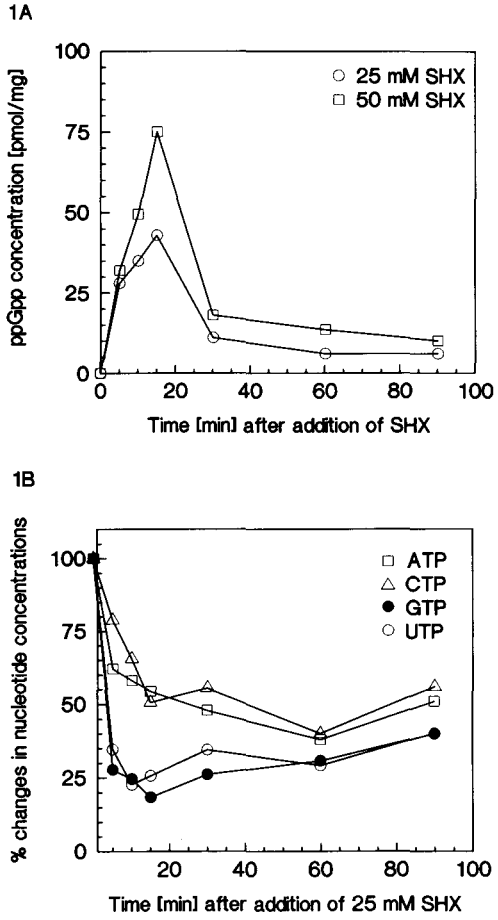


**Fig. 1.** A. ppGpp concentrations after addition of SHX to exponentially growing cultures ($OD_{450\,nm}$ = 0.35) of *S. coelicolor* A3(2) strain M145. B. Relative changes in nucleoside triphosphate concentrations after addition of SHX. The initial concentrations (0 min), estimated from dry weights of mycelium, were: ATP, 3294 pmol $mg^{-1}$; UTP, 1895 pmol $mg^{-1}$; CTP, 1050 pmol $mg^{-1}$; GTP, 1274 pmol $mg^{-1}$.

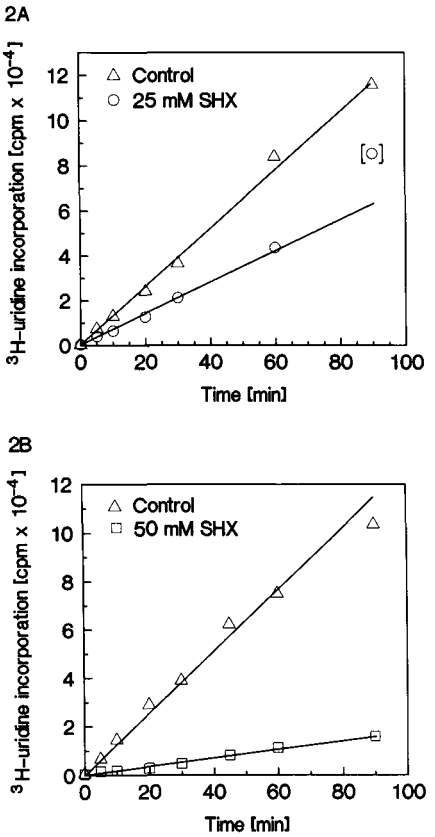


**Fig. 2.** Total RNA synthesis after addition of SHX to exponentially growing cultures ($OD_{450\,nm}$ = 0.35) of *S. coelicolor* A3(2) strain M145. RNA synthesis was determined by incorporation of [5,6-$^3$H]-uridine into TCA-precipitable material. The 90 min point in Fig. 2A was not considered when drawing the line.

*rrnD* transcripts (T. M. Clayton, personal communication).) Since all of the primary *rrnD* transcripts are processed at the same rate (Clayton and Bibb, 1990), the amounts of RNA-protected probe observed after S1 nuclease treatment presumably reflect the relative strengths of the promoters *in vivo*. In exponentially growing control cultures, p3 and p4 were the stronger promoters (Fig. 3), with initiation at p1 and p2 accounting for less than 10% of transcripts; transcription from p2 was barely detectable. The strongest band, which constituted approximately 70% of the RNA-protected counts, represents the premature form of 16S rRNA (indicated as PrS (processing site) in Fig. 3). Within 20 min of addition of 50 mM SHX the amounts of each of the four primary transcripts fell to below 10% of their initial values (Fig. 3A). Similar, but less dramatic, effects were observed with 25 mM SHX, with transcripts from p1, p3 and p4 falling to approximately 30% of their initial value (transcription from p2 was too low to be reliably quantified, but certainly decreased). These results suggest that all four of the *rrnD* promoters are subject to negative stringent control; assuming there was no differential effect on processing of the primary transcripts, then all four promoters appeared to be affected equally upon induction of the stringent response.

### *Induction of stringent response in* S. coelicolor *A3(2) by nutritional shiftdown*

The stringent response in *S. coelicolor* A3(2) was also induced by transferring exponentially growing cultures ($OD_{450\,nm}$ = 0.5) from an amino-acid-containing medium into a true minimal medium, as in Ochi (1986; 1987a,b,c). In contrast to the SHX experiments, the cultures showed little continued growth, with only a slight increase in optical density before they entered stationary phase. The changes in nucleotide concentrations were remarkably different from those observed after the addition of SHX. ppGpp accumulation was much more pronounced, giving a maximum value of 192 pmol mg$^{-1}$ dry weight 10 min after shiftdown (Fig. 4A). A small transient accumulation of pppGpp (guanosine 5′-triphosphate-3′-diphosphate) was also observed 10 min after amino acid depletion. Another new nucleotide was detectable in the high-performance liquid chromatography (HPLC) spectra that showed the same kinetics of appearance as ppGpp; a peak with identical characteristics was obtained by alkaline hydrolysis of ppGpp, suggesting that it might be ppGp (guanosine 5′-diphosphate-3′-monophosphate) which occurs during the stringent response in *E. coli* (magic spot III; Pao and Gallant, 1979). While ATP, UTP and CTP showed increased concentrations that persisted for at least 60 min after shiftdown, which presumably reflected the decrease in RNA synthesis, the GTP level fell dramatically to 20% of its initial value (Fig. 4B). The drop in GTP concentration far exceeds the amount converted into ppGpp; this might indicate another role for GTP during the stringent response. Protein synthesis, estimated by the incorporation of [$^{14}$C]-leucine into TCA-precipitable material, was also severely reduced.

Chloramphenicol binds to the large subunit of the ribosome and inhibits the formation of peptide bonds, thus preventing the uncharging of aminoacyl tRNAs and suppressing ppGpp production (Davis and Ron, 1980). When 5 μg ml$^{-1}$ chloramphenicol was added to M145 cultures, growth was reduced, but not prevented, presumably indicating partial inhibition of protein synthesis; when added immediately before shiftdown, ppGpp formation 10 min after shiftdown was reduced from 192 pmol mg$^{-1}$ dry weight to 25 pmol mg$^{-1}$ dry weight, with RNA synthesis showing a partially relaxed phenotype (Fig. 5).

After nutritional shiftdown, there was a dramatic decrease in transcript initiation from all of the *rrnD* promoters (Fig. 6); within 20 min, transcripts initiating from p1, p3 and p4 fell to approximately 10% of their initial levels. The

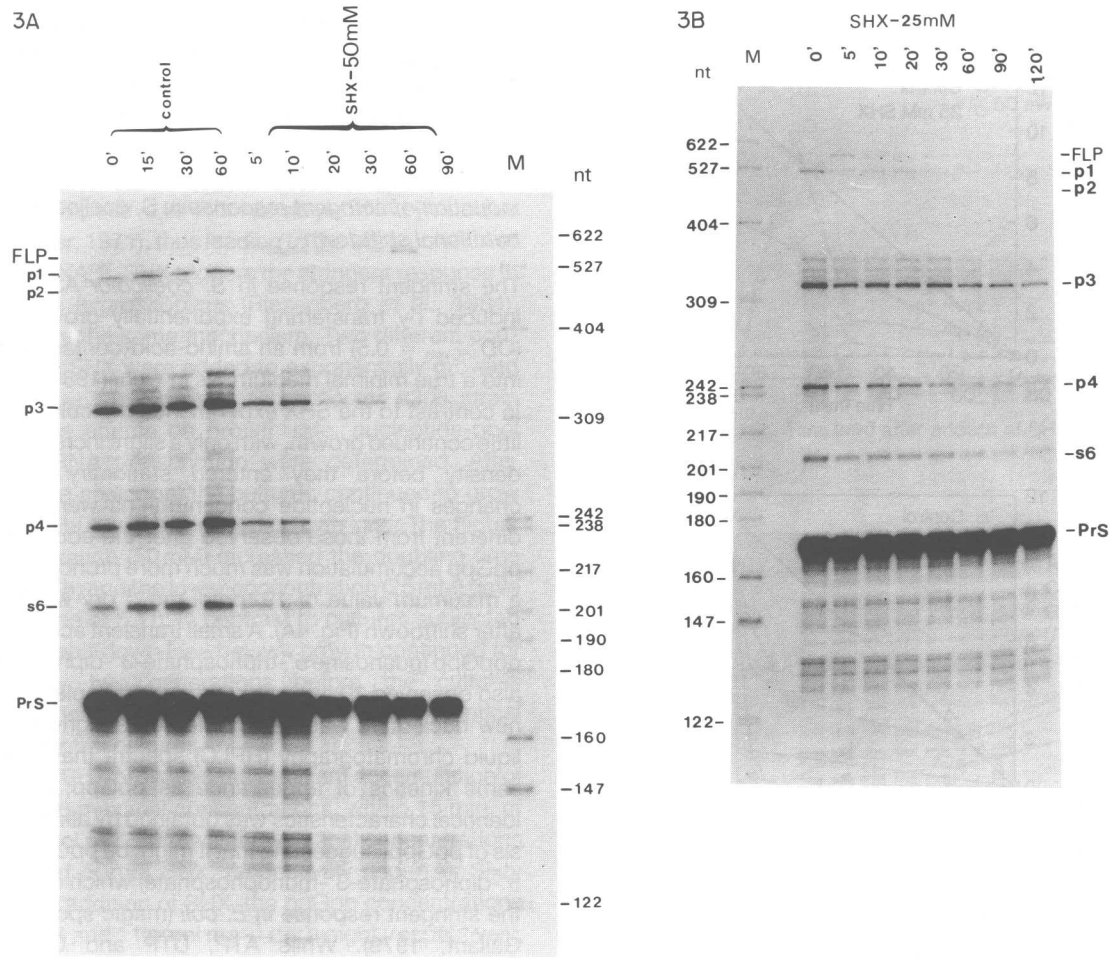


**Fig. 3.** S1 nuclease mapping of the promoters of the *rrnD* gene set of *S. coelicolor* A3(2) strain M145. p1, p2, p3 and p4 represent promoter transcripts, s6 is an unassigned RNA-protected band (see text), PrS indicates the RNA-protected fragment derived from the processing site, and FLP marks the location of the full-length probe. End-labelled *Hpa*II-digested pBR322 (lane M) was used as size marker. Control RNA samples were isolated from untreated cultures. The times indicate minutes after addition of SHX to appropriate exponentially growing cultures at an $OD_{450\,nm}$ of 0.35.

RNA-protected band s6, which probably stems from one of the other five rRNA gene sets of *S. coelicolor* A3(2) (Baylis and Bibb, 1988b), also appeared to be under negative stringent control (Fig. 6A).

### *ppGpp and antibiotic production in* S. coelicolor *A3(2)*

Studies on different *Streptomyces* strains have led to the proposition that ppGpp is a key signal metabolite in initiating antibiotic biosynthesis (Ochi, 1986; 1987a,b,c). To assess this possibility in *S. coelicolor* A3(2), where further genetic analysis would be facilitated, we initially determined ppGpp concentrations throughout a normal batch culture and attempted to correlate any changes in these levels with the onset of production of actinorhodin and undecylprodigiosin, two pigmented antibiotics made by this strain (Hopwood, 1988). In the medium used in this study, *S. coelicolor* A3(2) strain M145 produces actinorhodin and undecylprodigiosin only when the cultures enter stationary phase. Undecylprodigiosin was detectable as soon as the cultures stopped growing, whereas actinorhodin production could be detected only six to eight hours later (Fig. 7A). Production of both antibiotics continued for several days, rising to 300 μg $ml^{-1}$ actinorhodin and 5 μg $ml^{-1}$ undecylprodigiosin after 120 h. ppGpp could not be detected in the early and mid-exponential growth phases (detection level was 3 pmol $mg^{-1}$ dry weight; Fig. 7A), but increased levels were observed at the end of exponential growth (16 pmol $mg^{-1}$ dry weight) and fell to 5–7 pmol $mg^{-1}$ dry weight during stationary phase. To determine when transcription of the actinorhodin biosynthetic genes commenced, the expression of *act*III, an early gene of the actinorhodin cluster that encodes a β-ketoreductase (Hallam *et al.*, 1988), was

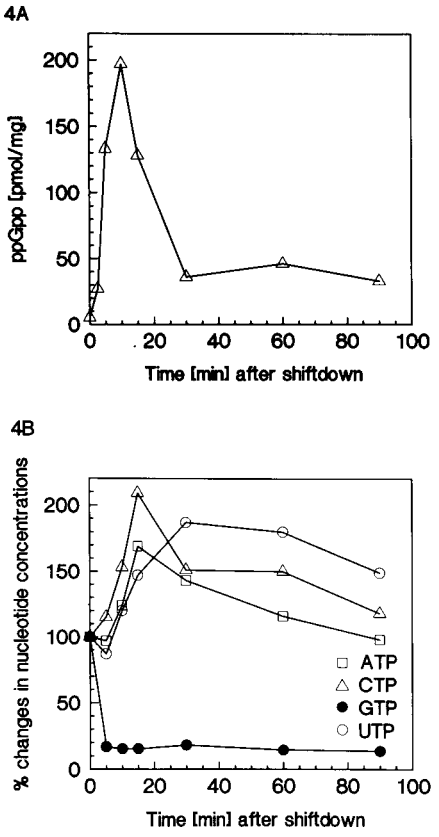


**Fig. 4.** ppGpp concentrations after nutritional shiftdown of an exponentially growing culture ($OD_{450\,nm}$ = 0.5) of *S. coelicolor* A3(2) strain M145.
B. Relative changes in nucleoside triphosphate concentrations after nutritional shiftdown. Initial values (0 min), estimated from dry weights of mycelium, were: ATP, 2709 pmol $mg^{-1}$; UTP, 692 pmol $mg^{-1}$; CTP, 732 pmol $mg^{-1}$; GTP, 874 pmol $mg^{-1}$.

studied by S1 nuclease mapping. An *act*III transcript corresponding to that identified by Hallam *et al.* (1988) was observed immediately after the culture had entered stationary phase (Fig. 7B).

We also assessed ppGpp levels and *act*III transcription after inducing the stringent response. After a nutritional shiftdown, the ppGpp concentration reached a maximum level of approximately 200 pmol $mg^{-1}$ dry weight within 10 min and fell to a level of about 6 pmol $mg^{-1}$ dry weight. The cultures increased only slightly in optical density (from $OD_{450\,nm}$ = 0.5 to 0.85) before effectively entering stationary phase approximately four hours after shiftdown (Fig. 8A). Although actinorhodin was not measurable until 10 h after inducing the stringent response, *act*III transcripts were detected one hour after shiftdown (Fig. 8B). In contrast to the untreated cultures, and to those treated with SHX (see below), actinorhodin and undecylprodigiosin production appeared to coincide; the reason for this difference is not known.

ppGpp levels and transcription of *act*III were also determined after induction of the stringent response with 25 mM SHX. In contrast to the nutritional shiftdown, the culture continued to grow exponentially at a reduced rate for two generations (Fig. 9A). ppGpp accumulated to 47 pmol $mg^{-1}$ dry weight before falling to 6 pmol $mg^{-1}$ dry weight. A small transient increase in ppGpp to about 10 pmol $mg^{-1}$ dry weight occurred again at the end of exponential growth. *act*III transcripts were detected only when the cultures had entered stationary phase, approximately 12 h after induction of the stringent response (Fig. 9B).

### *Isolation of a relaxed mutant of* S. coelicolor *A3(2)*

Relaxed mutants, apparently equivalent to *relC* mutants, have been isolated at frequencies of 3–10% among spontaneously thiostrepton-resistant mutants of a number of *Streptomyces* species, and are deficient in antibiotic production (Ochi, 1986; 1987a,b,c; 1988). Isolates of *S. coelicolor* A3(2) strain M145 that were resistant to 25 μg $ml^{-1}$ thiostrepton were therefore assessed for a relaxed phenotype after nutritional shiftdown. Whereas in M145, total RNA synthesis fell by 75% 30 min after shiftdown, one of the thiostrepton-resistant mutants, M145-R1, showed a reproducible reduction of RNA synthesis of only 55% 30 min after shiftdown (Fig. 5). Furthermore, this mutant produced only around 20% (43 pmol $mg^{-1}$ dry weight) of the level of ppGpp found in M145 after amino-acid starvation. However, although M145-R1 resembles the apparent *relC* mutants isolated by Ochi both in RNA synthesis and in ppGpp production

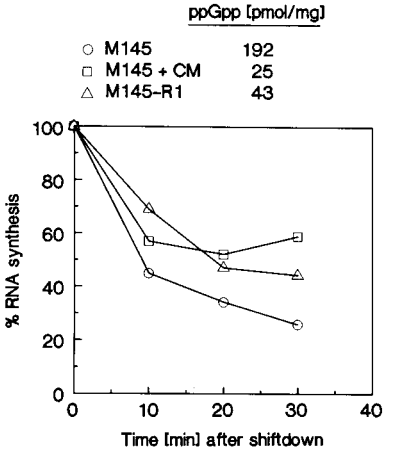


**Fig. 5.** Total RNA synthesis after nutritional shiftdown of exponentially growing cultures ($OD_{450\,nm}$ = 0.5) of *S. coelicolor* A3(2) strain M145, M145 in the presence of 5 μg $ml^{-1}$ chloramphenicol, and M145-R1; the maximal observed levels of ppGpp after shiftdown are shown on the right. RNA synthesis was determined by incorporation of (5,6-$^3$H)-uridine into TCA-precipitable material and expressed as a percentage of the counts obtained with untreated control cultures. CM, chloramphenicol.

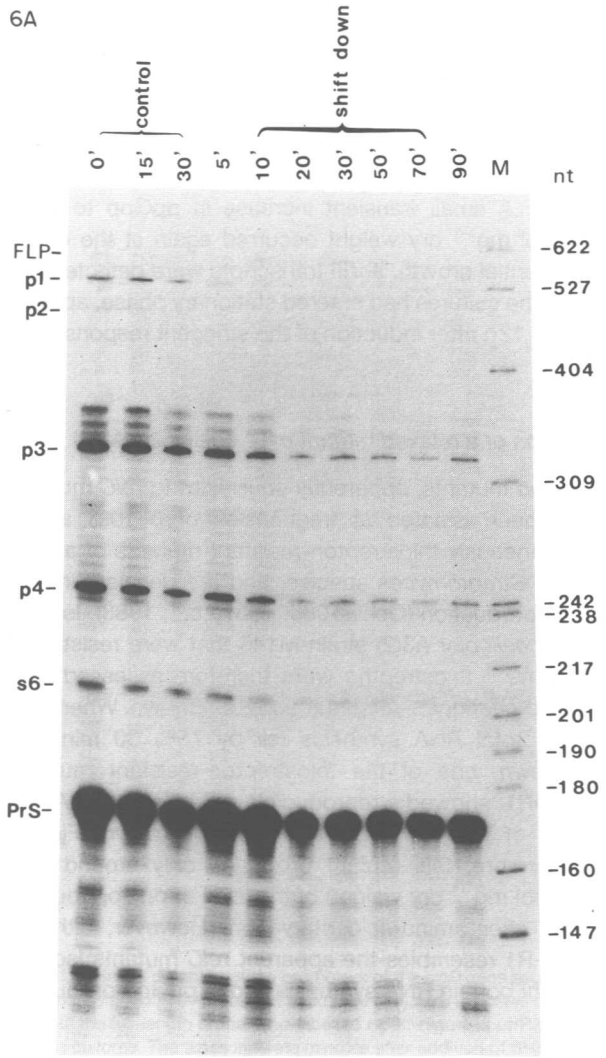


**Fig. 6.** A. S1 nuclease mapping of the promoters of the *rrnD* gene set of *S. coelicolor* A3(2) strain M145 after nutritional shiftdown of exponentially growing cultures ($OD_{450\,nm}$ = 0.5; see the legend to Fig. 3 for details). The apparent reduction in band intensities in the 30 min control sample reflect a lower sample loading and is not physiologically significant.
B. Quantitative analysis of the p1, p3 and p4 transcript abundances by gel scanning.

after shiftdown, it had a normal growth rate, suggesting that it is not a typical *relC* mutant. The time course and amounts of actinorhodin and undecylprodiogiosin produced, both with and without nutritional shiftdown, closely resembled those of the parental strain.

## Discussion

Two different methods, nutritional shiftdown and addition of serine hydroxamate, were used to provoke the stringent response in *S. coelicolor* A3(2): both appeared to trigger the formation of ppGpp. The accumulation of ppGpp was significantly lower when SHX was used. Riesenberg *et al.* (1984) previously used SHX to elicit the stringent response in *Streptomyces hygroscopicus* and observed relatively small ppGpp accumulations (19 pmol $mg^{-1}$ dry weight with 20 mM SHX). The nutritional shiftdown described by Ochi (1986; 1987a,b) proved a more efficient way to trigger ppGpp formation; the ppGpp accumulations that we observed in *S. coelicolor* A3(2) (around 200 pmol $mg^{-1}$ dry weight) were in the same range as those reported by Ochi for other *Streptomyces* strains, which varied from 82 pmol $mg^{-1}$ in *Streptomyces antibioticus* (Ochi, 1987a) to 800 pmol $mg^{-1}$ in *Streptomyces griseus* (Ochi, 1987b). Such observations are consistent with previous work in *Bacillus subtilis,* where starvation for many amino acids triggered more pronounced changes in nucleotide concentrations than starvation for just one (Lopez *et al.*, 1981).

The growth of *S. coelicolor* A3(2) was affected differently by the two treatments. While the SHX-treated cultures underwent two doublings before reaching stationary phase, those subjected to nutritional shiftdown showed little further growth. The changes in intracellular nucleoside triphosphate concentrations were also remarkably different. In SHX-treated cultures, their concentrations fell to lower basal levels, while in the nutritional shiftdown experiments, with the exception of GTP, transient increases in concentrations occurred. The kinetics of both sets of changes are in reasonable agreement with the data of Riesenberg *et al.* (1984) and Ochi (1986; 1987b).

Although the amount of data is limited, there is a correlation between the peak levels of ppGpp accumulation and the reduction in both growth rate and total RNA synthesis. Such results are reminiscent of the situation in *E. coli*, where ppGpp appears to play a central role in growth-rate control (Sarubbi *et al.*, 1988); perhaps ppGpp plays a similar role in *Streptomyces*.

Discarding the unlikely possibility that inducing the stringent response affected the rate of processing, the stringent response in *S. coelicolor* A3(2) led to a marked reduction in rRNA transcription, as it does in *E. coli* (Gallant, 1979). However, in contrast to *E. coli*, all of the *rrnD* promoters appeared to be subject to negative stringent control and all appeared to be affected equally.

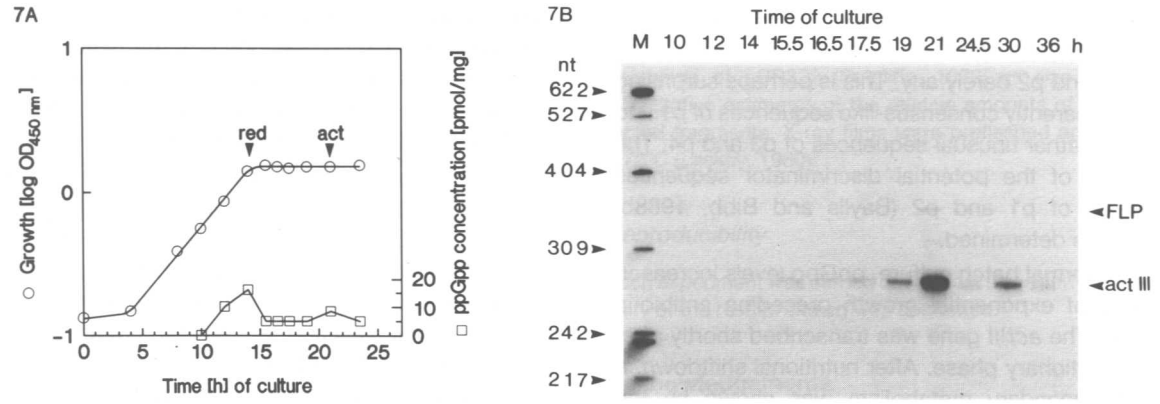


**Fig. 7.** A. ppGpp levels during a normal batch culture of *S. coelicolor* A3(2) strain M145. Arrows indicate when undecylprodigiosin (red) and actinorhodin (act) were first detected using the spectrophotometric assays.
B. S1 nuclease mapping of *act*III of *S. coelicolor* A3(2) strain M145. RNA was isolated at the times indicated (see the legend to Fig. 3 for details).

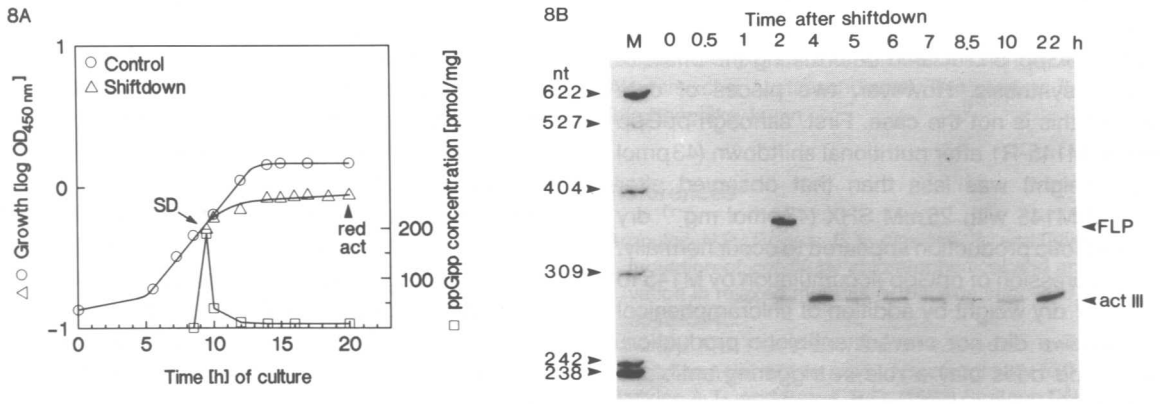


**Fig. 8.** A. Growth of *S. coelicolor* A3(2) strain M145 after or without nutritional shiftdown, and ppGpp production by the shifted culture. The arrow indicates when actinorhodin (act) and undecylprodigiosin (red) were first detected spectrophotometrically in the culture subjected to shiftdown.
B. S1 nuclease mapping of *act*III after nutritional shiftdown. RNA was isolated at the times indicated (see the legend to Fig. 3 for details).

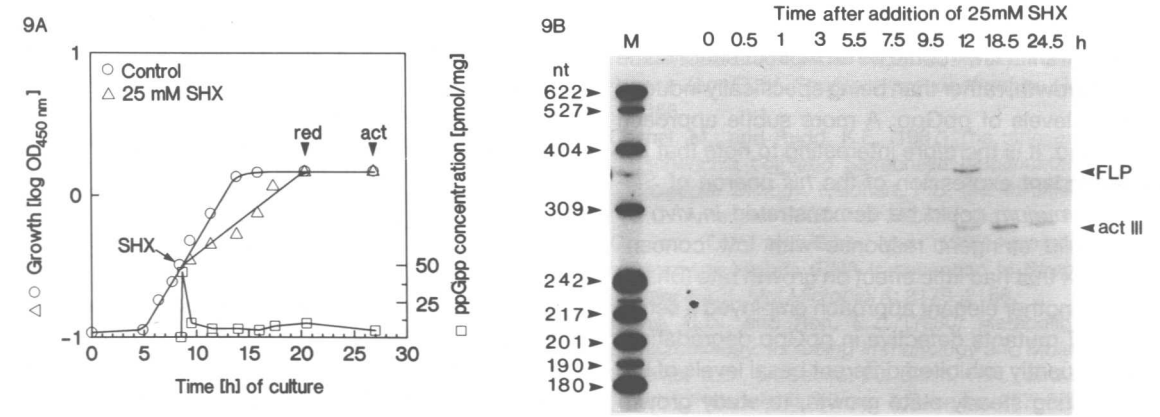


**Fig. 9.** A. Growth of *S. coelicolor* A3(2) strain M145 after or without addition of 25 mM SHX, and ppGpp production by the treated culture. Arrows indicate when actinorhodin (act) and undecylprodigiosin (red) were first detected spectrophotometrically in the treated culture.
B. S1 nuclease mapping of *act*III after SHX addition. RNA was isolated at the times indicated (see the legend to Fig. 3 for details).

During exponential growth the major promoters were p3 and p4, with p1 contributing about 10% of all *rrnD* transcripts, and p2 barely any. This is perhaps surprising given the apparently consensus-like sequences of p1 and p2, and the rather unusual sequences of p3 and p4. The role, if any, of the potential discriminator sequences downstream of p1 and p2 (Baylis and Bibb, 1988b) remains to be determined.

During a normal batch culture, ppGpp levels increased at the end of exponential growth preceding antibiotic production. The *act*III gene was transcribed shortly after entry into stationary phase. After nutritional shiftdown, a switch to secondary metabolism was shown by the appearance of the *act*III transcript one hour later. After the SHX-induced stringent response, exponential growth continued and although there was a transient accumulation of ppGpp, *act*III transcripts were detected only 12 h later, when the cultures entered stationary phase. Since ppGpp production after SHX treatment was significantly lower than after nutritional shiftdown, it might appear that the level of ppGpp is crucial in determining the onset of antibiotic biosynthesis. However, two pieces of data suggest that this is not the case. First, although ppGpp synthesis in M145-R1 after nutritional shiftdown (43 pmol $mg^{-1}$ dry weight) was less than that observed after treatment of M145 with 25 mM SHX (47 pmol $mg^{-1}$ dry weight), antibiotic production appeared to occur normally. Second, suppression of ppGpp accumulation by M145 to 25 pmol $mg^{-1}$ dry weight by addition of chloramphenicol before shiftdown did not prevent antibiotic production. Thus, if ppGpp does play a role in triggering antibiotic biosynthesis, it would appear that it is not by itself sufficient to promote this transition.

Although nutritional shiftdown leads to high levels of ppGpp, complete starvation for amino acids arrests growth and, at least in *E. coli*, provokes a variety of global stress responses (Van Bogelen *et al.*, 1987). This makes it difficult to assess the precise role of ppGpp in regulating cell physiology. For example, the transcription of *act*III after nutritional shiftdown could be an indirect effect of the cessation of growth, rather than being specifically induced by increased levels of ppGpp. A more subtle approach may be needed. It is therefore interesting to note that the ppGpp-dependent expression of the *his* operon of *Salmonella typhimurium* could be demonstrated *in vivo* by inducing a mild stringent response with low concentrations of SHX that had little effect on growth rate (Shand *et al.*, 1989). Another elegant approach employed a series of *E. coli spoT* mutants defective in ppGpp degradation, which consequently exhibited different basal levels of the metabolite during steady-state growth, to study growth rate and *rrnA* ribosomal promoter regulation (Sarubbi *et al.*, 1988). Similar approaches that permit changes in ppGpp levels in the absence of severe physiological trauma may be required to elucidate the role of ppGpp in regulating antibiotic production in *Streptomyces.*

An inverse correlation between growth rate and the level of ppGpp has been reported for *E. coli* (Sarubbi *et al.*, 1988). Thus, although the elevated level of ppGpp that occurs in *S. coelicolor* A3(2) at the end of the exponential growth and that precedes the onset of antibiotic production is intriguing, it may simply reflect the decrease in growth rate. The proposition that ppGpp plays an important role in the initiation of antibiotic biosynthesis relies heavily on the isolation of relaxed mutants that are deficient in antibiotic production (Ochi, 1986; 1987a,b). However, the inability of such mutants to produce antibiotics is not necessarily associated with their reduced ability to form ppGpp. In *Bacillus subtilis*, a *relC* mutation led to a conformational change in ribosome structure that prevented the ribosome from translating the mRNA of the chloramphenicol acetyltransferase gene; the deficiency in ppGpp production was not responsible for the lack of expression of resistance (Ambulos *et al.*, 1988). Effects such as these, and the general effect of *relC* or other *rel* mutations on cell growth (Stark and Cundliffe, 1979), should be borne in mind when attempting to establish a causal relationship between the production of ppGpp and the onset of antibiotic production.

Our results suggest that elevated levels of ppGpp are not sufficient to initiate antibiotic biosynthesis in *S. coelicolor* A3(2). However, the transient increase of ppGpp at the end of exponential growth, and its occurrence in stationary-phase cultures, could indicate that it might be a requirement for the expression of genes involved in secondary metabolism.

## Experimental procedures

### *Bacterial strains, plasmids and culture conditions*

*S. coelicolor* A3(2) strain M145 ($SCP1^-$, $SCP2^-$; Hopwood *et al.*, 1985) was grown in a modified form of NMP minimal liquid medium (Hopwood *et al.*, 1985) that contained 0.2% (w/v) casamino acids (instead of 0.5%), and 1 mM $KH_2PO_4$ and 25 mM TES-buffer, pH 7.2 (instead of 15 mM $NaH_2PO_4/K_2HPO_4$, pH 6.8). Preliminary experiments indicated that actinorhodin and undecylprodigiosin production by M145 in this medium was not subject to carbon (glucose), nitrogen ($NH_4SO_4$) or phosphate ($KH_2PO_4$) repression. High-titre spore suspensions were obtained on R2YE plates (Hopwood *et al.*, 1985) and pre-germinated for 6–8 h in 2 × TY medium (Bankier and Barrel, 1983). Estimates of growth were made by measuring optical density and dry weight, with the two methods giving a good correlation. Nutritional shiftdown was performed when cultures reached an $OD_{450nm}$ of 0.5. Cultures were harvested either by centrifugation or filtration (Whatman nitrocellulose filters, 0.45 μm pore size) and resuspended in modified NMP without casamino acids. D, L-serine hydroxamate (SHX; Sigma) was added to growing cultures at an $OD_{450nm}$ of 0.35. pIJ2820 (Baylis and Bibb, 1988b) and pIJ5104

(Hallam *et al.*, 1988) were used as sources of probes for S1 nuclease mapping of *rrnD* and *act*III transcripts, respectively. Actinorhodin production was determined spectrophotometrically after adjusting supernatants to pH12 with 1 N NaOH; an $OD_{608nm}$ of 0.5 corresponds to 60 μg $ml^{-1}$ actinorhodin (H. M. Kieser, personal communication). Undecylprodigiosin (molecular weight 393) was extracted with methanol from mycelium acidified with 1 N HCl and the absorption measured at 530 nm; the molar extinction coefficient at this wavelength is 100500 (Tsao *et al.*, 1985).

### *Extraction and quantification of nucleotides*

Mycelium from 50–100 ml cultures was harvested on nitrocellulose filters (Whatman, pore size 0.45 μm), extracted with cold 1 M formic acid for 1 h, centrifuged for 10 min at 8000 × *g* and the supernatants were filtered through nitrocellulose (Whatman, pore size 0.45 μm). After freeze-drying of the supernatants, samples were resuspended in 200–400 μl of $H_2O$. Nucleotide concentrations were determined by HPLC on a Partisil 10SAX column with a gradient of 7 mM $KH_2PO_4$, pH 4.0 to 0.5 M $KH_2PO_4$/0.5 M $Na_2SO_4$, pH 5.4 at a flow rate of 1.5 ml $min^{-1}$, as described by Ochi (1987c). Concentrations of nucleotides were determined relative to mycelial dry weight at the time of harvesting. Nucleotide standards were bought from ICN Biomedicals and Boehringer Mannheim.

### In vivo *labelling of RNA and RNA isolation*

For labelling of RNA *in vivo*, cultures were harvested by centrifugation or filtration on nitrocellulose filters (Whatman, pore size 0.45 μm) and resuspended in the same medium with or without casamino acids, supplemented with 2 μCi $ml^{-1}$ [5,6-$^3$H]-uridine (Amersham, 50 Ci $mmol^{-1}$) and unlabelled uridine (12.5 μg $ml^{-1}$; 51 mM). Samples were withdrawn at the indicated times and pipetted into cold 5% TCA. Samples were filtered through Whatman GF/C discs, dried, and counted. RNA was isolated according to the method of C. P. Smith (Hopwood *et al.*, 1985); RNA concentrations were determined spectrophotometrically and the quality of the preparations was checked by gel electrophoresis.

### *High-resolution S1 nuclease mapping*

High-resolution S1 nuclease analysis (Sharp *et al.*, 1980) was performed according to Bibb *et al.* (1986) with the following modifications. For the *rrnD* transcripts, 1 μg of RNA was hybridized to 0.15 pmol (approximately $10^5$ Cerenkov c.p.m.) of a 570 bp *Acc*I–*Ban*I fragment containing the leader region of *rrnD* uniquely labelled at the 5′ end of the *Acc*I site (Baylis and Bibb, 1988b) in 20 μl of NaTCA buffer (Murray, 1986). NaTCA was made according to Summerton *et al.* (1983). Hybridization was carried out at 45°C for 6 h after denaturation at 65°C for 30 min. For the *act*III transcripts, 30 μg of RNA was hybridized to 0.02 pmol (approximately 1.5 × $10^4$ Cerenkov c.p.m.) of a 354 bp *Bam*HI–*Sal*I fragment that contained the promoter and 5′ coding regions of *act*III (Hallam *et al.*, 1988) and that was uniquely labelled at the 5′ end of the *Sal*I site. Hybridizations were performed in 20 μl of formamide buffer (Hopwood *et al.*, 1985) at 57°C for 6 h after denaturation at 85°C for 30 min. RNA-protected fragments were electrophoretically resolved on 6% (w/v) polyacrylamide/7 M urea gels (Sanger and Coulson, 1978) using end-labelled *Hpa*II fragments of pBR322 (Sutcliffe, 1979) as size markers. For quantitative estimates of the relative amounts of the RNA-protected fragments, X-ray films were preflashed and exposed at −70°C (Laskey, 1980).

### *Reproducibility*

Each experiment was carried out at least twice and the reproducibility of the results shown was confirmed.

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